Mapping the Regulatory Role of the HD-ZIPIII START Domain Across Plant Evolution

Research Thesis

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by

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Abstract

To determine if the regulatory function of the START domain has been conserved across the plant kingdom, a diverged CLASS III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) protein from Klebsormidium flaccidum was transformed into Arabidopsis thaliana to observe misexpression gain-of-function phenotypes. However, observation of no misexpression phenotype coupled with confocal imaging of confirmed accumulation and nuclear localization of the protein suggests Klebsormidium HD-ZIP III is non-functional in Arabidopsis. Three potential explanations are explored, and a new experiment is proposed to circumvent this short-coming.

Introduction

Plants share a variety of conserved genes called transcription factors (TFs) that coordinate and regulate development and maintain homeostasis through the regulation of their target gene expression. The predominant thought in the field is that the primary driver of developmental evolution are changes to cis-regulatory regions that TFs bind which in turn alter their targets expression pattern in a context-dependent manner (Carrol 2005; Wray 2007). Therefore, evolutionary biologists have given a lot of attention to how changes in these regions affect transcriptional levels and patterns. However, coding sequence evolution can also be considered a driver of developmental evolution (Wolpert et al 2015; Galis et al 2007; Carrol 2005). It is thought that cis-regulatory regions are more tolerable to mutations which in-turn alter expression patterns. This compared to the coding region in which mutations can lead to harmful pleiotropic effects and is, therefore, more constrained and static. But studies have shown that gene

duplications and whole genome duplications can allow for these constraints to be circumvented (Galis 2007; Soltis et al 2016; Marchant et al 2019). Specifically, duplication of a gene can allow for divergence in the coding region, thus changing its function without depriving the organism of the essential function of the original gene. In animals, the amplification and diversification of TFs, specifically the nuclear receptor class of TFs (NRs), positively correlate with developmental complexity (Sladek 2011). It is, therefore, useful to investigate how TFs amplification and coding sequence evolution affect developmental processes.

The HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP) super-family of TFs are found throughout the plant kingdom (Goodstein et al., 2012; Romani et al., 2017). They are defined by the presence of a homeodomain, which allows for DNA binding, and their leucine zipper domain, which allows for homo-dimerization. One sub-family, the CLASS III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIPIII), is an excellent example of duplication events leading to diversification and novel developmental processes. HD-ZIPIIIs have been co-opted and redeployed in land plant developmental innovations including cell identity in lateral organs, stem cell maintenance in the shoot apical meristem (SAM) as well as the root apical meristem (RAM), and xylem identity in vasculature tissue (McConnell et al., 2001; Husbands et al., 2009; Carlsbecker et al., 2010). HD-ZIPIII TFs are thus a potential candidate to explore how coding sequence evolution affects complex developmental processes.

HD-ZIPIIIs have 4 domains in total: the homeodomain, leucine zipper domain, putative lipid binding START domain, and the MEHKLA domain (Fig. 1 A) and are post-transcriptionally regulated by microRNA (miRNA) 165/166 (McConnell et al., 2001). START domains are activated through high-affinity binding to lipophilic ligands (Ponting and Aravind, 1999; Tsujishita et al., 2000; Roderick et al., 2002). The presence of a homeodomain coupled with a

lipophilic binding domain is a hallmark of mammalian NRs. Although no orthologs of mammalian NRs are found in plants, HD-ZIPIIIs may be functionally-analogous and represent a new class of NRs (Lumba et al., 2010). If true, this suggests an underlying mechanism by which complex developmental processes evolve. This, therefore, warrants further investigation into the role of the HD-ZIPIII START domain activity on the protein.

Unpublished data from our lab shows that the HD-ZIPIII START domain is in the "classical" family of START domains. Homology modeling of the HD-ZIP III START domain shows that it closely resembles phosphatidylcholine transfer protein (PCTP)(Fig. 1 B; Husbands et al, unpublished). In conjunction with PCTP and other classical START domains, putative ligand contacting residues have been predicted (Husbands et al, unpublished). In unpublished data from our lab, the START domain present in the HD-ZIPIII protein PHABULOSA (PHB) has been shown to regulate the activity of this protein (fig. 1 C; Husbands et al, unpublished). miRNA166 resistant PHB produces a range of gain-of-function (GOF) phenotypes, including radialized leaves and enlarged meristems (McConnell et al., 2001). Mutating or deleting the START domain abolishes those GOF phenotypes, indicating that the START domain plays a role in regulating HD-ZIPIII activity.

We want to further investigate the regulatory role of this domain, and perhaps reveal a general mechanism by which organisms create complex developmental patterns. We will do this by using orthologs of various HD-ZIPIII proteins and assessing whether START contribution to HD-ZIPIII protein activity is conserved across the plant kingdom.

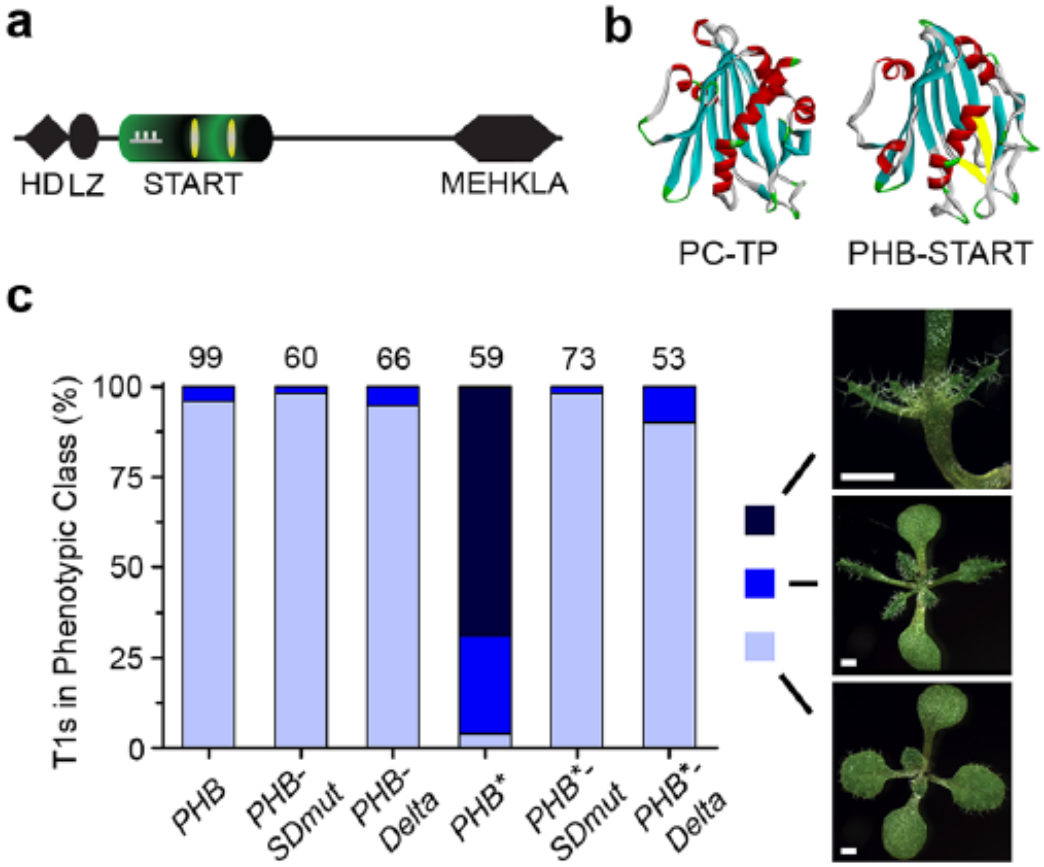
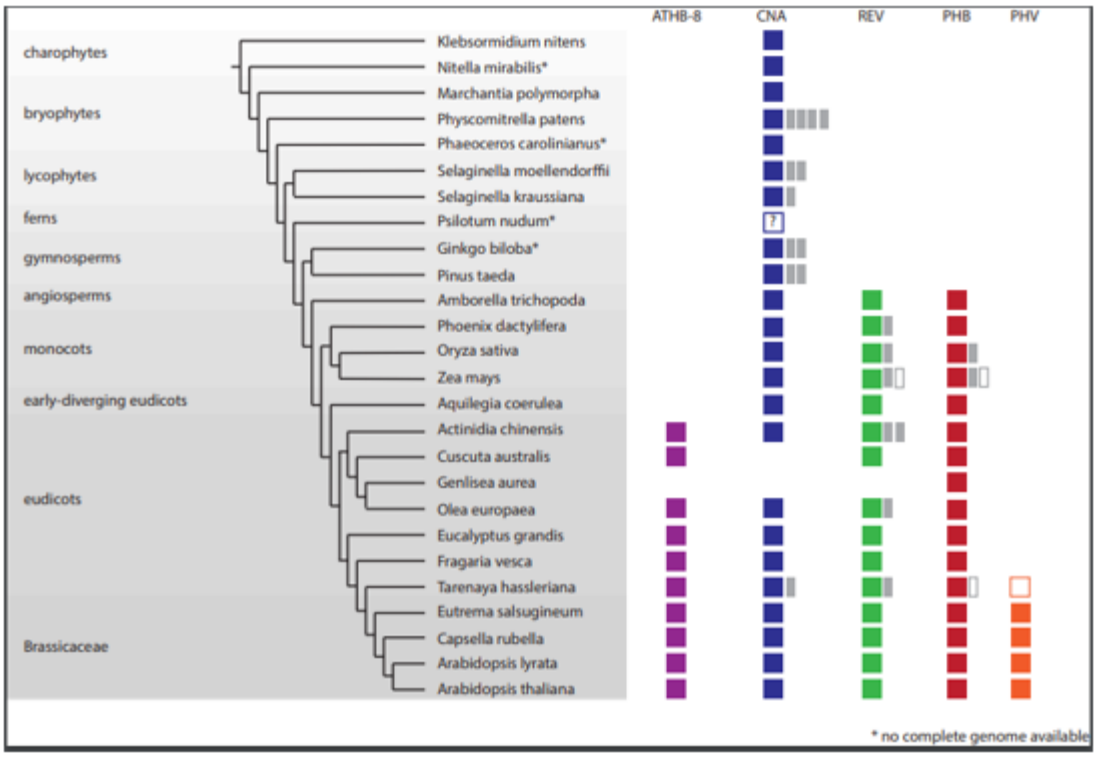


Fig 1. The START domain is required for PHB function. **a)** Structure of a general HD-ZIP III. Yellow areas represent mutated residues. The comb represents miRNA binding site. **b)** PHB-START (right) resembles PC-TP (left). Yellow areas represent mutated residues in PHB-SDmut lines. **c)** PHB transformants phenotypically tallied. Light blue indicates normal phenotypes. Dark blue indicates moderate phenotypes. Black indicates severe phenotypes. PHB variants sensitive to miRNA show normal phenotypes. The miRNA resistant (*) PHB is ectopically expressed and shows severe and moderate phenotypes. When miRNA resistant strains START domain is mutated (PHB-SDmut) or deleted (PHB-Delta), transformants appear wild-type.

Results

HD-ZIPIII TFs arose with multicellularity (Goodstein et al., 2012; Romani et al., 2018). They are not found in single cellular algae but are found in their multicellular descendants. Klebsormidium nittens, a multicellular alga, contains only one HD-ZIPIII protein (fig. 2). As more complex developmental innovations arose, so too do more HD-ZIP III TFs appear. Although there isn't a direct correlation with complex development and number of HD-ZIPIII TFs, this does seem to be a general trend with a few exceptions.

Arabidopsis thaliana contains five paralogs of the HD-ZIP III family: PHABULOSA (PHB), PHAVULOSA (PHV), REVOLUTA (REV), CORONA (CNA), and ATHB8 (fig. 2). All five proteins have both redundant and unique activities. The most diverged extant species from Arabidopsis with an HD-ZIPIII is Klebsormidium flaccidum (Kleb), a filamentous multicellular alga. To assess whether the putative lipid binding START domain has always been necessary for HD-ZIP III protein function, we first decided to express the coding region of Klebs HD-ZIP III (KFC3HDZIP) in Arabidopsis to determine if it too gives a gain-of-function phenotype similar to PHB in Arabidopsis.



Katia Andrianova

Figure 2. Phylogenetic Tree of Arabidopsis five HD-ZIP III paralogs. The five HD-ZIP III paralogs found in Arabidopsis thaliana are represented by five different colored boxes (right). Blue represents CORONA (CNA). Green represents REVOLUTA (REV). Red represents PHABULOSA (PHB). Purple represents ATHB-8. Orange represents PHAVULOSA (PHV). Grey boxes represent paralogs within the species. Phylogenetic tree (left) shows major families of the plant lineage. CNA represents the most ancestral-like of the HD-ZIP III paralogs found in Arabidopsis. There is a general trend of increased complexity and number of HD-ZIP III present in the genome with a few exceptions.

KFC3HDZIP transgene

In order to express KFC3HDZIP in Arabidopsis, an RNA extraction was performed on Klebsormidium tissue and cDNA was synthesized. The cDNA was attached to the promoter

pREV (the native promoter for REV), REV 5' UTR for regulatory elements, Citrine YFP in order to determine accumulation and expression pattern, a 3x FLAG to perform biochemical analysis, and the 3' IGR of REV in case of any regulatory sequences downstream (fig. 3). Although KFC3HDZIP is most similar to CNA, the promoter and regulatory regions of REV were used as it has the broadest expression pattern of the Arabidopsis HD-ZIPIII paralogs. This is not an overexpression, however, as REV is expressed at similar levels as CNA. The fragments were stitched together and inserted into the cloning vector pCR8 using a Gibson Assembly reaction.

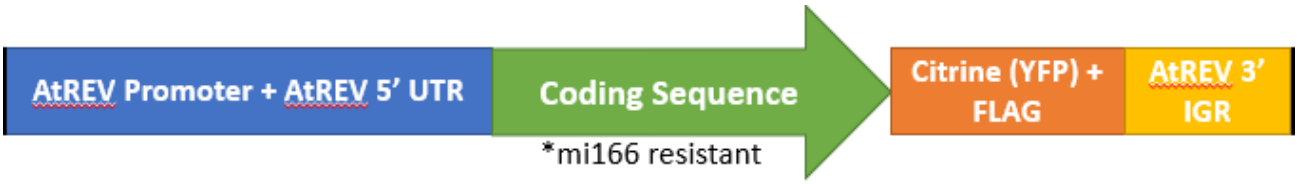


Figure 3. KFC3HDZIP Transgene. (Left to Right) The native promoter of Arabidopsis REV attached to its 5' untranslated region (UTR) (blue), KFC3HDZIP coding sequence (green arrow), Citrine YFP attached to 3xFLAG (orange), REV 3' inter-genomic region (IGR).

Chi-Square Analysis

The transgene pREV-KFC3HDZIP was then transferred into the binary vector pB7GW using an LR reaction, electroporated into Agrobacterium tumefaciens, and then transformed into Arabidopsis via floral dip. Plants were selected for with Basta (pB7GW confers Basta resistance), and after selection, 10 independent transgenic lines were propagated for downstream analyses. Approximately 100 T1 seeds from each line were grown on MS0-PPT plates for selection (pB7GW grants PPT resistance). A chi-square analysis was performed to determine the number of transgene insertions into the genome (table 1). This makes analyses easier as it minimizes variation in protein production. All but two lines, numbers 1 and 8, were within the

parameters of a single insertion. These seedlings were then transplanted to soil to grow and be phenotypically analyzed.

KFC3HDZIP	Alive	Dead	X^2	P-Value
Line 1	69	35	4.16	≈.05
Line 2	79	19	2.46	≈.1
Line 3	81	24	.25	≈.9
Line 4	72	24	0	≈.995
Line 5	122	38	.13	≈.9
Line 6	67	26	.44	≈.5
Line 7	74	23	.08	≈.9
Line 8	72	43	9.29	≈.005
Line 9	108	36	0	≈.995
Line 10	101	24	2.24	≈.5

Table 1. X^2 analysis of KFC3HDZIP transformants. Transformed plants were plated onto MS0-PPT plates for selection. Expected segregation of T2 generation with one insertion of the transgene is 3:1 (alive: dead). Lines 1 and 8 were shown to fall outside of the expected ratio (p-values ≤ .05). The other 8 lines were used to continue with the phenotypic analysis.

Misexpression phenotypes

Because misexpression produces a GOF phenotype, all plants misexpressing the protein should show obvious phenotypic characteristics such as radialized leaves, deformed roots, or enlarged meristems. Images from the Ochando lab (Ochando et al., 2006) were used to compare phenotypes between our pREV-KFC3HDZIP to their CNA misexpression lines (fig.4 A-C

Ochando et al. 2006). Although some phenotypes were similar to those shown by the Ochando lab, e.g. indented leaves with curled-up edges, the inconsistency suggests that KFC3HDZIP is not producing a GOF phenotype. Other typical HD-ZIPIII misexpression phenotypes were also investigated (images not shown). The majority of the transformed plant's meristems looked WT as well as no noticeable deformities in root morphology. These data suggest that KFC3HDZIP may not be functional in Arabidopsis.

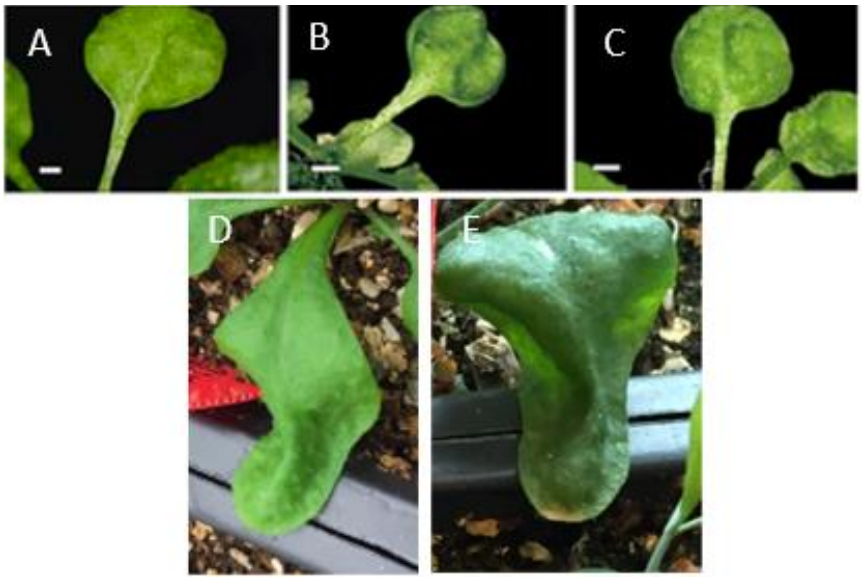


Figure 4. Phenotypic observation and comparison of HD-ZIPIII misexpression. a) Wild-type Arabidopsis leaf (21 days old). The leaf is flat with no signs of morphological deformity. **b and c)** CNA misexpression phenotype from the Ochanda lab (21 days old). Indents in the leaves and curling-up of the edges represent a moderate leaf morphological deformity. **d and e)** KFC3HDZIP misexpression lines. No noticeable phenotypes until later in development (35 days old). Indents in the leaf are seen but no curling-up of the edges. Phenotypes are inconsistent and therefore KFC3HDZIP is considered inactive. Images A-C are republished from Ochando et al, 2006.

Confocal imaging

One possible reason for the lack of phenotypes in our transgenic lines is that protein is not accumulating. To test this idea, confocal imaging was used to look at KFC3HDZIP in the root tip of lines shown to have only one insertion of the transgene. Confocal imaging indicates KFC3HDZIP is indeed accumulating (fig. 4 A), eliminating the possibility of protein degradation. Another possible explanation is that KFC3HDZIP is not localized to the nucleus in Arabidopsis, an essential requirement for TF funtion. However, confocal imaging showed that KFC3HDZIP does localize to the nucleus. Images from the Xie lab (Xie et al. 2014) were used to compare pREV-KFC3HDZIP-YFP expression pattern to pREV-REV-GFP expression pattern (fig. 5 B). Native REV is also found localized to the nucleus in the medial portion of the root. Comparison of the two shows no difference in expression pattern. Thus KFC3HDZIP protein accumulates in Arabidopsis and is nuclear localized, but it does not condition GOF phenotypes in Arabidopsis.

Panel B is republished from Xie et al. 2014

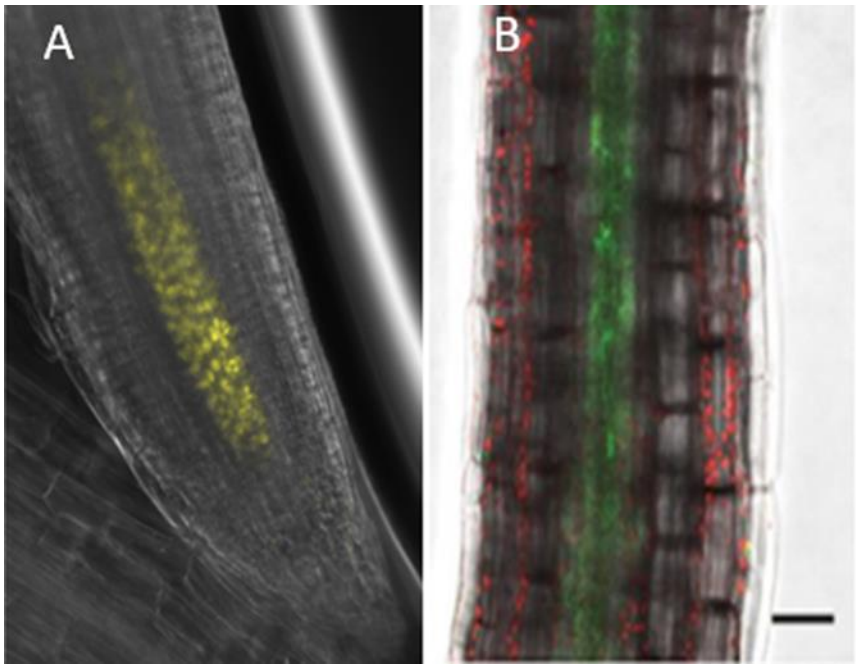


Figure 5. Confocal imaging of Arabidopsis roots. a) Confocal analysis of KF3HDZIP-YFP shows accumulation and nuclear localization in root tip of Arabidopsis. **b)** Image from the Xie lab showing accumulation and nuclear localization of REV-GFP. Xie lab gives no explanation for RFP expression.

Discussion

The confocal analysis shows proper folding of KFC3HDZIP in root tips of Arabidopsis but shows no obvious phenotypes when looking at morphological features of the transformed plants. There are a few potential explanations as to why KFC3HDZIP is not producing GOF phenotypes in Arabidopsis.

First, KFC3HDZIP may not be able to bind Arabidopsis DNA, or bind to cis-elements in different targets. A multiple sequence alignment (MSA) was made to look at the homeodomain of the five HD-ZIPIII paralogs and KFC3HDZIP (fig 6.) DNA contacting residues of the five paralogs are known and conserved. As seen in the MSA, these contacting residues are also conserved in KFC3HDZIP. In fact, there is high conservation towards the C-terminal side of the homeodomain, leading us to believe that contacting different cis-elements is an unlikely explanation. However, it has been shown that proteins with high conservation in domains but low conservation in other areas of the proteins affect domain function (Weinber et al., 2017). The low conservation seen in the N-terminal region of the protein may inhibit binding of the conserved contacting residues.

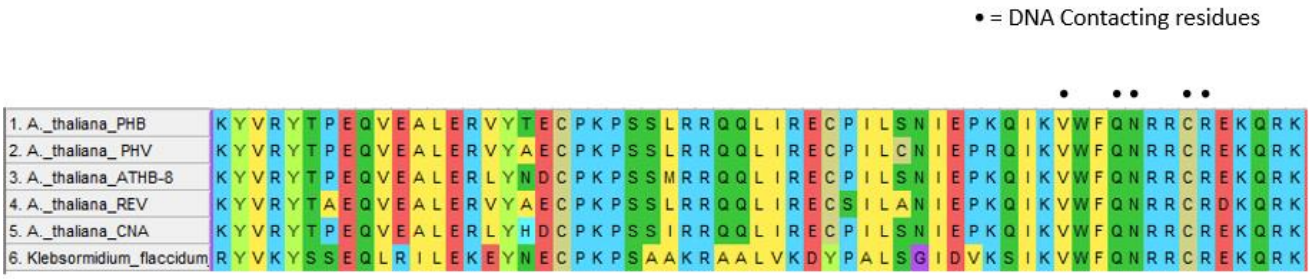


Figure 6. Multiple Sequence Alignment of the HOMEODOMAIN of Arabidopsis thaliana five paralogs and KFC3HDZIP. DNA contacting residues (•) are conserved in all the homeodomains. C-terminal side shows high conservation while the N-terminal side shows variation.

Another possible explanation is the inability of KFC3HDZIP to interact with necessary protein partners for transcription. Unpublished data from our lab shows that in Arabidopsis, HD-ZIPIII TFs interacts with several proteins, such as chromatin remodelers, in order to activate transcription (data not shown). If these interactions are not taking place, even if KFC3HDZIP is binding DNA, no transcription would occur thus no production of misexpression phenotypes.

The final explanation I provide is that KFC3HDZIP ligand is not found in Arabidopsis. Unpublished data suggest that when an HD-ZIP III binds its ligand, it undergoes a conformational change that allows for DNA binding by its homeodomain. If the ligand is not present in Arabidopsis, KFC3HDZIP would be locked in its inactive state and thus be unable to produce GOF phenotypes.

In order to investigate KFC3HDZIP further, we will have to circumvent these pitfalls. One way to do so is to perform a domain swap between KFC3HDZIP START domain and the START domain of CNA. Professional computational genomics collaborator, Dr. Igor Jouline, identified

CNA as the ortholog of KFC3HDZIP. This will allow us to still assess the role of KFC3HDZIPs START domain without confounding effects from the rest of the protein sequence. Since CNA is native to Arabidopsis, it should have no problem binding DNA or interacting with its protein partners. However, if the KFC3HDZIP ligand isn't found in Arabidopsis, then the domain swap will be fruitless. To overcome this pitfall, KFC3HDZIP ligand will have to be identified.

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Materials and Methods

Synthesis of KFC3HDZIP cDNA

Ashton Holub extracted KFC3HDZIP mRNA by collecting, flash-freezing, and grinding Kleb tissue. He followed a trizol/chloroform RNA extraction protocol and stored collected samples at -80˚C until needed.

For synthesis of cDNA, 1ug of RNA from Kleb, 1uL of oligo dT, 1uL of dNTP at 10mM were added to a PCR tube and raised to 10uL with RNase free water. The tube was placed in a thermocycler and heated to 65˚C for 5 minutes to remove secondary structures. The PCR tube was then removed from the thermocycler. Next, 4uL MgCl2 at 25mM concentration, 2uL 10X RT Buffer, 2uL DTT at 0.1M concentration, 1uL RNaseOUT, and 1uL Superscript III Reverse Transcriptase were added to the tube.

The PCR tube was placed in the thermocycler at 50˚C for 1 hour. The content was then transferred to a microcentrifuge tube and 20uL of water was added. Using the reverse primer (5'-GCTCACCATGTCGACTCCACCTCCACCTCCTTGGTAGTGCAGCAGCG-3') and forward primer (5'-TTTGAGACTTTTTTTGAGGGTCGAGCTAAAATGGGGGGCAGCGAG-3') KFC3HDZIP was amplified using PCR. Q5 protocol of: 21.5uL water, 10uL 5X Q5 reaction buffer, 10uL 5X Q5 high GC enhancer, 1uL dNTPs at a concentration of 10 mM, 2.5uL forward primer, 2.5uL reverse primer, 2uL of cDNA, and 0.5uL Q5 polymerase. Thermocycler program: 98˚C for 30 seconds, denature at 98˚C for 8 seconds, anneal at 67˚C for 30 second, extend at 72˚C for 2 minutes. Repeat 35x, 72˚C for 2 mintues. Hold at 4˚C

Construction of KFC3HDZIP Transgene

To acquire the REV promoter, 5'UTR, and 3'IGR, Arabidopsis genomic DNA was isolated, and PCR was used to amplify the regions. Two separate reactions were done; one fore the REV promoter and 5'UTR the other for REVs 3'IGR. This is because the promoter and 5'UTR are adjacent in the genome while the 3'IGR is further downstream. For the first reaction, forward primer (5'- tctatgtagtttaaacttttttggataattc-3') and reverse primer (5'- tttagctcgaccctcaaaaaaagtctcaaa-3') were used in the PCR reaction: 22.5 uL water, 10uL 5X Q5 reaction buffer, 10uL 5X Q5 high GC enhancer, 4uL dNTP at a concentration of 2.5mM, 2.5uL forward primer and reverse primer, 1uL genomic DNA, and 0.5 uL Q5 polymerase. Thermocycler program used: 98˚C for 30 seconds, denature at 98˚C for 8 second, anneal at 60˚C for 30 seconds, extend at 72˚C for 120 seconds. Repeat 33x. 72˚C for 2 minutes and then hold at 4˚C. The second reaction utilized forward primer 5' – ttcgattgacagaaaaagactaatttaaat -3'and reverse primer 5'- cttctctctttacacgcccaaaatcgttgaagt -3'. The same PCR reaction and thermocycler program were used as described above. Citrine-YFP and 3XFLAG were available from Aman Husbands from previous experiments.

To stich together the five fragments and insert into the cloning vector pCR8, the Gibson Ultra assembly protocol was used. DNA fragments, including pCR8, were diluted in nuclease-free water in PCR tubes to a total volume of 5uL. 5uL of GA Ultra master mix A (2X) was pipetted into the PCR tube containing the fragments and plasmid. Tubes were then vortexed and span down. Tubes were then placed in thermocycler. The program used was: 37˚C for 5 minutes, inactivated 75˚C for 20 minutes, slowly cooled by .1˚C/sec until 60˚C, annealed at 60˚C for 30 minutes and then slowly cooled by .1˚C/sec to 4˚C. 10uL of GA Ultra master mix B (2X) was then added. The solution was mixed by pipetting. The reaction was then incubated at 45˚C for 15 minutes.

Construction of KFC3HDZIP-pCR8 Plasmid

Using electrocompetent E. coli, Gibbson assembled KFC3HDZIP transgene inserted into pCR8 was transformed into via electroporation. 2uL of the assembled transgene was added to thawed electrocompetent E. coli. Using a electroporation machine, was used to shock the E. coli into taking up

the pCR8 plasmid. E. coli was left to rest and then plated on LB Spec plates for selection. Colonies were allowed to grow for amplification of the plasmid. Plasmids were then isolated, and a restriction digest was used to confer proper insertion into pCR8. Restriction enzyme used was EcoR1. Electrophoresis of a 1% agarose gel was used to assess digested plasmids. UV imaging showed correct banding pattern of digested pCR8 plasmid.

To confer correct nucleotide sequence of transgene, sequencing primers were ordered and used to perform Sanger Sequencing. Eleven reactions were done for each candidate (4 candidates in total). Reactions were sent off to the Genomics Shared Resource facility at OSU. Two candidates came back correct. One of the candidates was used to continue with experiments downstream.

LR Clonase Reaction for Construction of KFC3HDZIP-pB7GW Plasmid

In order switch the transgene from pCR8 into the binary vector pB7GW, an LR reaction was performed. In a microcentrifuge tube, 4uL of pCR8 with transgene insertion, 1uL of destination vector pB7GW, 3 uL of TE buffer, and 2uL of LR Clonase II enzyme was added and microcentrifuged briefly. Reaction was incubated at 25˚C for 24 hours. 1uL of proteinase K solution was added and vortexed briefly. Reaction was incubated at 37˚C for 1 hour. The solution was transformed into E. coli using the electroporation protocol described above. Transformed E. coli was then plated onto LB spec plates for selection. Colonies were collected allowed to grow for plasmid isolation. The plasmid was isolated and correct insertion was tested for by a restriction enzyme digest with Spa1 and EcoR1. Using electrophoresis, the digested plasmid was checked for correct banding pattern.

Isolated plasmids that were confirmed were then used to transform into Agrobacterium via electroporation. 0.5 uL of intact plasmid was added to tube of Agrobacterium. Using the electroporation machine, the cells were zapped and the allowed to rest for 3 hours. Transformed cells were plated onto LB Spec Kan plates for selection. The transformed cells were incubated at 26˚C for 2 days. Colonies were then collected and grown for another two days for plasmid isolation. Using miniprep, pB7GW

plasmids were isolated and tested for correct integration by restriction digest using Spa1 and EcoR1. Colonies with correct banding pattern were then used to continue into dipping.

Arabidopsis Dipping

Agrobacterium with pB7GW were allowed to grow in a 500mL Erlenmeyer flask for two days. Using a beaker, 500mL of distilled water, with 25g of sucrose and 250uL of Tween were added together to make the dipping solution. The 500mL of agrobacterium were split into 2 250mL tubes and centrifuged to create a pellet of cells. The supernatant was poured-off and the cells resuspended in the 250mL of the dipping solution. The 2 250mL of resuspended cells were poured into a pan. Wt Arabidopsis were then dipped into the pan. A pipette was used to drop the solution onto the meristem, floral organs, and any other part of the plant that did not get touched when dipped into the pan. The plants were put on their side and covered for 1 day to rest. The plants were re-dipped the following day and allowed to rest again.

Confocal imaging of KFC3HDZIP in Arabidopsis Roots

Using a confocal microscope, 8-day old T2 Arabidopsis roots were pulled, wet mounted to a microscope slide, and imaged. Using YFP/CFP dichromic mirror and path-cube, KFC3HDZIP was able to be imaged. The laser for YFP was at 30% while the laser for CFP was kept at 0%. The gain used of the YFP channel was 7.2 to reduce background fluorescence.